# THE REGULATION OF INTERFERON PRODUCTION BY ASPIRIN, OTHER INHIBITORS OF THE CYCLOOXYGENASE PATHWAY AND AGENTS INFLUENCING CALCIUM CHANNEL FLUX*

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THE interferon family of proteins is an important component of the immune system. Three major types of human interferon are known to exist and these have been designated interferon types alpha, beta, and gamma. All three types share the ability to exert significant antiviral properties, but gamma interferon also seems to be an important immune modulator.[1] Conceptually, interferon may itself be thought of as a system with afferent and efferent components. The afferent limb consists of those factors which lead to the production of interferon and the efferent limb as those factors which ultimately permit interferon to assert its biological effects.

Just as with other components of the immune system, pharmacological agents can modulate the interferon system. Thus, we and others have shown in the past that corticosteroids[2-4] and antimetabolites[3] can influence the production of human interferon gamma and the latter can have similar effects on the production of human interferon alpha. Likewise, we have found that antimetabolites can diminish the antiviral effects of interferon.[5] We have therefore been interested in other agents that might influence this important antiviral defense network and particularly in those drugs that might enhance the production or bioactivity of interferon and possibly compensate for the adverse effects of some of the other agents we have studied.

Prostaglandins are among a group of natural body substances known to

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influence the production of human interferon gamma.[6,7] Furthermore, indomethacin,[6] an inhibitor of prostaglandin synthesis, has been reported to enhance the production of human interferon gamma. It seemed important, therefore, further to explore the effects of inhibitors of prostaglandin synthesis on the interferon system. Similarly, calcium and calcium dependent intracellular enzyme systems have important influences on the integrity of the immune system, and it seemed reasonable to determine whether manipulation of calcium or the calcium dependent intracellular systems could also exert regulatory influences on the interferon system. With these questions in mind we undertook a series of investigations, some of which have previously been published elsewhere,[8,9] and which we will review here.

## METHODS

The methods utilized here have previously been published in detail.[8,9] Briefly, peripheral blood mononuclear cells were obtained from healthy blood donors from the blood bank. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation and stimulated with one of several inducers (PHA, IL 2 or ionomycin) for the induction of human interferon gamma, or with complexed polyinosinic- polycytidylic acid (poly rI:rC) for the induction of human interferon alpha. Various agents described were added independently to tubes containing the inducers and titers of interferon obtained were compared with those found in similar tubes simultaneously prepared and treated identically except for the fact they did not contain the test drug.

Interferon assays were performed in microtiter using a bioassay that employs WISH cells and a viral challenge of either vesicular stomatitis virus or encephalomyocarditis virus. Appropriate toxicity controls were employed in every test and cell viability determined by exclusion of trypan blue.

Whenever human interferon gamma was produced, proliferative responses were also determined utilizing uptake of tritiated thymidine.

## RESULTS

Initial studies using polyclonal antibodies verified that interferon produced in human peripheral blood mononuclear cells after incubation with either PHA, IL 2, or ionomycin was indeed human interferon gamma and that induced in the same peripheral blood mononuclear cells after incubation with poly rI:rC was human interferon alpha.

Table I demonstrates the effect of acetylsalicylic acid on the production of human interferon gamma. From this table it can be seen that in the presence

TABLE I. ACETYLSALICYLIC ACID EFFECT ON THE PRODUCTION OF HUMAN INTERFERON

| *Treatment* | *IFN titer** | *P*** *Value* |
|---|---|---|
| Cells alone | <3 | — |
| PHA† | 8.3±0.4 | — |
| PHA+ASA (1.0 ugm/ml) | 9.3±0.5 | >.20 |
| PHA+ASA (10.0 ugm/ml) | 10.1±0.4 | <.05 |
| PHA±ASA (100.0 ugm/ml) | 8.5±0.5 | >.10 |
| Poly rI:rC‡ | 8.0±0.5 | — |
| Poly rI:rC+ASA (1.0 ugm/ml) | 9.0±0.6 | >.10 |
| Poly rI:rC+ASA (10.0 ugm/ml) | 9.5±0.6 | <.02 |
| Poly rI:rC+ASA (100.0 ugm/ml) | 9.2±0.6 | >.10 |

*Expressed as the $Log_2$ of the mean titer ± standard error of the mean
**P value for the comparison between the titer observed with the inducer alone and this titer
†Mean of 16 experiments
‡Mean of 14 experiments

of acetylsalicylic acid, yields were enhanced. However, only at the 10 ugm/ml concentration was the yield of human interferon gamma significantly greater than the yields found using PHA without the acetylsalicylic acid. In separate experiments not shown here, it was established that acetylsalicylic acid alone did not induce secretion of human interferon gamma. It should also be emphasized that the data from which Table I was derived included a total of 16 individual experiments utilizing peripheral blood mononuclear cells from eight different donors. Proliferative responses performed on these cells also indicated that the enhanced yields of human interferon gamma were associated with increased uptake of tritiated thymidine and hence cellular proliferation. Similar experiments were also performed using other inhibitors of prostaglandin synthesis, mainly ibuprofen and indomethacin, and again increased yields of human interferon gamma of approximately similar proportions were found.

In Table I we can also see the effect of acetylsalicylic acid on the production of human interferon alpha. Again we note that its presence was associated with increased production of interferon, which was significantly greater at the 10 ugm/ml concentration. Approximately similar increases were seen with ibuprofen and indomethacin.

Since we had demonstrated a definite effect of acetylsalicylic acid on the production of both species of human leukocyte interferon, we were anxious to be sure of the mechanism responsible for this. We next explored the influence of prostaglandins on the production of human interferon by peripheral blood mononuclear cells. It can be seen (Table I) that prostaglandin E2

TABLE II. THE EFFECT OF PROSTAGLANDINS ON THE PRODUCTION OF HUMAN INTERFERON GAMMA

| *Treatment* | *IFN titer** |
|---|---|
| Cells alone | >3 |
| PHA | 8.0 ± 1.2 |
| PHA + PGA1 (0.1 ugm/ml) | 7.3 ± 1.4 |
| PHA + PGA2 (0.1 ugm/ml) | 7.5 ± 1.2 |
| PHA + PGF1 (0.1 ugm/ml) | 7.5 ± 1.3 |
| PHA + PGF2 (0.1 ugm/ml) | 7.7 ± 1.4 |
| PHA + PGE2 (0.1 ugm/ml) | 5.3 ± 1.4 |

*Expressed as the $Log_2$ of the mean titer ± standard error of four experiments

TABLE III. THE EFFECT OF PROSTAGLANDINS ON THE PRODUCTION OF HUMAN INTERFERON ALPHA

| *Treatment* | *IFN titer** |
|---|---|
| Poly rI:rC | 7.0 + 0 |
| Poly rI:rC + PGA1 (0.1 ugm/ml) | 7.7 + 1.5 |
| Poly rI:rC + PGA2 (0.1 ugm/ml) | 8.5 + 1.7 |
| Poly rI:rC + PGAF1 (0.1 ugm/ml) | 8.5 + 1.7 |
| Poly rI:rC + PGAF2 (0.1 ugm/ml) | 7.5 + 1.7 |
| Poly rI:rC + PGE2 (0.1 ugm/ml) | 8.5 + 1.8 |

*Expressed as the $Log_2$ of the mean IFN titer ± the standard error

was associated with a decrease in the titer of human interferon gamma produced but that none of these agents diminished the yields of human interferon alpha (Table III). Using concentrations of prostaglandins one tenth of those seen in Table III, (i.e., 0.01 ugm/ml) we obtained identical results. Finally, we added the higher concentration of prostaglandins to the cultures of peripheral blood mononuclear cells along with the 10 ugm/ml acetylsalicylic acid. When present simultaneously, we were able to demonstrate that only the presence of PGE 2 negated the enhancing effect of the acetylsalicylic acid. In contrast, none of the prostaglandins diminished its effect on the production of human interferon alpha.

Measurement of total prostaglandins in cultures of peripheral blood mononuclear cells stimulated with PHA in the presence and absence of acetylsalicylic acid failed to show any significant differences, reemphasizing that only certain of the prostaglandins influenced the production of interferon by these cells.

Additionally, measurement of IL 2 in cultures of peripheral blood mononuclear cells stimulated with PHA in the presence or absence of acetylsalicylic acid did show changes similar to those observed with human interferon gamma. Thus, the presence of acetylsalicylic acid did enhance the secretion of IL 2 as well.

While all of the supernatants obtained from cultures of peripheral blood mononuclear cells were dialyzed to remove any pharmacological agents tested, we nonetheless wanted to be sure that residual drug did not in any way influence our results. Therefore, we assayed recombinant human gamma and alpha interferons in the presence and absence of both acetylsalicylic acid and prostaglandins and compared the resulting titers. None of the agents tested were found to affect the interferon titer.

Prostaglandins are often generated in the cell as a by-product of membrane changes following the interaction of various signals with receptors on the cell membrane.[10] These same cell membrane perturbations also lead to intracellular calcium fluxes that may activate other cellular processes.[10] We therefore began a series of experiments to determine the influence of the calcium messenger system on the generation of human leukocyte interferons. These experiments are currently being reported in detail.[9] Briefly, we confirmed that PHA stimulation of lymphocytes generates inositol trisphosphate and increases the intracellular concentration of calcium. Using specially prepared media free of calcium, we were able to show that the reduced calcium concentration in the media significantly diminished the yield of human gamma but not human alpha interferon.

To explore this phenomenon further, we used several pharmacological probes known to influence the flux of calcium into the cell (Table IV). First, we reconfirmed the work of Dianzani et al.,[11] and demonstrated that agents promoting the increase in intracellular calcium by enhancing the calcium flow through cellular membranes will stimulate human gamma interferon. We subsequently demonstrated that an agent promoting the flow of calcium through calcium channels, Bay K 8644,[12] will also induce the production of gamma interferon. These results can be seen in Table IV. Conversely, we examined the influence of blockade of the calcium channel on the production of both human interferon gamma and alpha (Table V). Using nifedipine, we demonstrated that the presence of channel blockers clearly diminished the production of human gamma interferon when PHA was used as the inducing or mitogenic agent; however, when IL 2 or ionomycin was used for this same purpose, no effect on interferon yields could be shown. Previously, Dianzani et al.[13] had shown that other calcium channel blockers had the same effect. When human interferon alpha was induced using poly rI:rC, no effect was demonstrated on yields. All agents were again removed by dialysis, but effects on biological activity of the interferon were again tested using the

TABLE IV. INDUCTION OF GAMMA INTERFERON BY IONOPHORES

| *Treatment* | *$Log_2$ IFN titer* | *Prolif. response CPM × $10^{-3}$* |
|---|---|---|
| Cells | 0 | 0.4 ± 0.3 |
| PHA | 8.4 ± 0.2 | 8.7 ± 3.9 |
| A23187 ($10^{-5}$M) | 7.7 ± 0.2 | 0.3 ± 0.3 |
| ($10^{-6}$M) | 8.0 ± 0.2 | 0.7 ± 0.4 |
| ($10^{-7}$M) | 6.7 ± 0.1 | 0.4 ± 0.1 |
| Ionomycin ($10^{-5}$M) | 7.6 ± 0.1 | 0.5 ± 0.5 |
| ($10^{-6}$M) | 6.9 ± 0.1 | 0.6 ± 0.3 |
| ($10^{-7}$M) | 6.7 ± 0.1 | 0.3 ± 0.1 |
| Bay K8644 ($10^{-5}$M) | 6.8 ± 0.1 | 0.3 ± 0.1 |
| ($10^{-6}$M) | 6.9 ± 0.1 | 0.4 ± 0.2 |
| ($10^{-7}$M) | 6.2 ± 0.1 | 0.5 ± 0.2 |

TABLE V. THE EFFECT OF NIFEDIPINE ON THE PRODUCTION OF HUMAN LEUKOCYTE INTERFERON

| *Inducing agent* | *$Log_2$ IFN* titer ± S.D.* | *P value*** | *Proliferative response* |
|---|---|---|---|
| PHA | 6.0 ± 1.0 | — | 5.6 ± 0.3 |
| PHA + Nifedipine $10^{-5}$ | <3.0 ± 0.0 | <0.05 | −2.6 ± 0.5 |
| PHA + Nifedipine $10^{-6}$ | 4.5 ± 0.7 | NS | 7.4 ± 1.1 |
| PHA + Nifedipine $10^{-7}$ | 5.5 ± 0.7 | NS | 7.4 ± 2.3 |
| IL-2 | 8.6 ± 0.6 | — | 3.3 ± 0.5 |
| IL-2 + Nifedipine $10^{-5}$ | 8.6 ± 0.5 | NS | 1.3 ± 0.3 |
| IL-2 + Nifedipine $10^{-6}$ | 8.0 ± 0.5 | NS | 2.6 ± 0.4 |
| IL-2 + Nifedipine $10^{-7}$ | 8.0 ± 1.0 | NS | 3.7 ± 0.6 |
| Ionomycin | 8.6 ± 0.5 | — | 5.4 ± 2.2 |
| Ionomycin + Nifedipine $10^{-5}$ | 8.7 ± 0.7 | NS | 4.4 ± 0.9 |
| Ionomycin + Nifedipine $10^{-6}$ | 8.3 ± 0.6 | NS | 2.9 ± 0.7 |
| Ionomycin + Nifedipine $10^{-7}$ | 8.7 ± 0.7 | NS | 3.9 ± 0.5 |
| Poly rI:rC | 7.0 ± 0.6 | — | — |
| Poly rI:rC + Nifedipine $10^{-5}$ | 8.3 ± 0.6 | NS | — |
| Poly rI:rC + Nifedipine $10^{-6}$ | 7.1 + 0.7 | NS | — |
| Poly rI:rC + Nifedipine $10^{-7}$ | 8.0 ± 0.7 | NS | — |

*Mean of 5 experiments

**P value for the mean IF titer with the inducer above or the inducer plus the designated concentration of nifedipine.

system employed above, and only A23187 was shown to affect interferon activity. A23187 enhanced bioactivity, as has been noted previously.[14]

## Discussion

These studies demonstrate the ability of acetylsalicylic acid to enhance the production of both human interferon alpha and gamma. Thus, in the presence of 10 ugm/ml of acetylsalicylic acid the yield of alpha interferon increased threefold and the yield of gamma interferon almost fourfold. In both cases this was a significant enhancement of production.

Since interferon itself has antiviral properties and particularly gamma interferon can augment host defenses such as macrophages,[1] it is quite possible that the enhanced secretion we have observed could provide an advantage to an individual with a viral infection if the in vitro observations we have made occur in vivo. Thus, the use of acetylsalicylic acid during the course of viral infections could do more than provide symptomatic relief.

The mechanism by which acetylsalicylic acid enhances the secretion of interferon is of considerable interest. Our data suggest that prostaglandin E2 can decrease production of gamma interferon, and replacement of prostaglandin E2 in cultures of acetylsalicylic acid-treated peripheral blood mononuclear cells negates the effect of the latter. These observations clearly suggest that inhibition of the secretion of certain of the prostaglandins is responsible for the enhanced secretion of interferon gamma. In contrast, prostaglandins did not seem to diminish the yield of alpha interferon in our studies, and addition of these agents to acetylsalicylic acid-treated peripheral blood mononuclear cells did not negate the effect of the aspirin in poly rI:rC stimulated lymphocytes. These observations may suggest that some other mechanism may account for the observed increase in the production of alpha interferon. Of interest is that Ikossi,[15] using sendai virus, found that prostaglandins El and E2 did diminish yields of human interferon alpha but Stroch et al.[16] found that these same compounds did not decrease interferon production by murine cell cultures in response to 10 carboxymethyl-9-acridanone.

It is further possible that the acetylsalicylic acid may render its interferon enhancing effect by increasing the yield of IL 2, which in turn may augment the secretion of the gamma interferon. Our data showing enhanced IL 2 yields in the presence of acetylsalicylic acid are compatible with this hypothesis as well.

Further attempts to enhance interferon yields led to exploration of membrane phenomenon occurring in stimulated cells. As pointed out above, prostaglandins can be a byproduct of certain of these cellular membrane events. Thus it seemed reasonable to determine what effects manipulation of certain of these events would have on the production of interferon. Initial studies clearly confirmed the fact that mitogenic stimulation of the peripheral blood mononuclear cells generated inositol trisphosphate and increased the concentration of intracellular calcium. We therefore decided to determine what effect alteration in cellular calcium fluxes might have on interferon yields. Simply diminishing the extracellular concentration of calcium significantly decreased the production of gamma but not alpha interferon. Addition of such calcium ionophores as ionomycin and A23187 induced interferon gamma in agreement with the results of others.[11] Addition of the calcium channel against Bay K 8644 similarly induced the secretion of interferon gamma. Finally, blockade of these channels with the use of nifedipine clearly reduced the yields of gamma but not alpha interferon, but this reduction depended on the nature of the inducer of the gamma interferon since nifedipine reduced gamma yields only when PHA was used as the inducing agent.

These data, like those above, suggest that the production of human interferon may be pharmacologically modulated. In some cases such as that of acetylsalicylic acid we can enhance secretion of both interferon alpha and gamma. In other situations, such as those involving calcium channel blockers, a decrease at least in the titer of gamma interferon may occur. It will now be of interest to determine whether the effects on interferon production observed with acetylsalicylic acid and nifedipine in cultures of peripheral blood mononuclear cells can be appreciated clinically.

## Summary

Interferon is a family of potent antiviral agents which can activate macrophages, enhance cell surface markers, or influence antibody production. Three major types of human interferon are known to exist and have been designated interferons alpha, beta, and gamma. Because of its unique antiviral properties and its ability to influence the immune response, interferon has long been considered a potential therapeutic intervention in the treatment of infections and possibly neoplastic diseases. Two potential means to utilize interferon might be considered: One method would involve the administration of exogenous interferon, but an alternative might augment natural interferon production. We have been investigating a series of pharmacological agents that might influence its production and action. Since prostaglandins influence the immune response, we have investigated the effect of these cyclic fatty acids and those agents that influence their production on soluble protein mediators of the immune response on interferon. Our studies have focused on the effects of acetylsalicylic acid on the interferon system. We have demonstrated that prostaglandins of the E series can significantly reduce the yields of human interferon gamma, but not alpha (the two species of leukocyte derived interferon). In general, yields of gamma interferon produced by peripheral blood mononuclear cells in the presence of $PGE_2$ (0.1 to

0.01 ugm/ml) were approximately 15% of those produced in the absence of these substances. In contrast, when acetylsalicylic acid (10 ugm/ml) was added to the cultures of peripheral blood mononuclear cells yields of gamma interferon increased more than threefold. When examining the effects of acetylsalicylic acid on human alpha interferon production, we were also to enhance interferon harvests although we could not demonstrate an adverse effect of prostaglandins on the production of these bioactive proteins. Addition of acetylsalicylic acid and prostaglandins simultaneously to our cultures had a negative effect on gamma interferon production, but still was associated with enhanced yields of interferon alpha.

In examining how prostaglandins might influence interferon production, we began to study other cellular requirements for lymphokine production including those processes which were calcium dependent. Preliminary studies demonstrated that production of human interferon gamma by peripheral blood mononuclear cells was calcium dependent, but production of human interferon alpha was not. Thus, almost all agents studied that influenced calcium dependent intracellular processes influenced the titer of human interferon gamma produced, but not that of human interferon alpha. In examining this phenomenon more closely we noted the calcium channel flux was critical to the production of interferon gamma, hence agents enhancing channel flux (Bay K 8644) increased the production of human interferon gamma, but agents diminishing channel flux (specifically) channel blockers diminished production of this interferon species. These effects depended on the nature of the specific inducing agent. We are now examining the relationship of our observations with calcium to our earlier work with acetylsalicylic acid.

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